FINAL REPORT

Prokaryotic cDNA Subtraction: A Method to Rapidly Identify Functional Gene Biomarkers

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Mary Jo Kirisits
Kerry A. Kinney
Susan K. De Long
University of Texas at Austin

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Table of Contents

List of Acronyms

cDNA	complementary deoxiyribonucleic acid
DNA	deoxyribonucleic acid
DoD	Department of Defense
ESTCP	Environmental Security Technology Certification Program
FXB	fixed-bed
MBT	molecular biology tools
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PRB	perchlorate-reducing bacteria
qPCR	quantitative polymerase chain reaction
RAP-PCR	ribonucleic acid arbitrarily primed polymerase chain reaction
RDA	representational difference analysis
RISA	ribosomal intergenic spacer analysis
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
RT-qPCR	reverse-transcription quantitative polymerase chain reaction
SERDP	Strategic Environmental Research and Development Program
SSH	suppression subtractive hybridization
T-RFLP	terminal restriction fragment length polymorphism
USEPA	United States Environmental Protection Agency

List of Figures

List of Tables

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Executive Summary

Perchlorate contamination of groundwater and surface water has been detected in at least 36 states and represents a public health concern. Bacteria capable of reducing perchlorate to chloride are ubiquitous in the environment, making biological perchlorate reduction an attractive treatment approach. For biological treatment to be successful, perchlorate-reducing bacteria (PRB) must not only be present, but they must also synthesize the enzymes that catalyze perchlorate reduction. The synthesis of specific enzymes, termed gene expression, is often regulated by each cell in response to environmental conditions (e.g., influent water characteristics). Changes in the composition or activity of the microbial community can lead to declines in perchlorate removal and ultimately, if left unchecked, failure of the treatment process.

Molecular biology tools (MBT) can be used to determine how the composition and activity of microbial communities change in response to operational parameters and to gain insight into the causes of process upsets. While many studies have sought to quantify particular groups of bacteria by targeting their 16S rRNA genes, this approach is not ideal for PRB because they are phylogenetically diverse. MBT that target functional genes (e.g., genes that encode biodegradation enzymes), might prove more useful for determining the capabilities of the bacterial communities and for predicting process performance. Functional genes can be interrogated at the level of deoxyribonucleic acid (DNA) to determine functional potential or at the level of ribonucleic acid (RNA) to determine functional activity.

The main objective of this research effort was to assess the utility of MBT to improve our understanding of and our control over biological perchlorate reduction. A range of MBT were investigated including terminal restriction fragment length polymorphism (T-RFLP), Prokaryotic suppression subtractive hybridization (SSH) polymerase chain reaction (PCR) complementary deoxyribonucleic acid (cDNA) Subtraction [1], reverse-transcription PCR (RT-PCR), and quantitative PCR (qPCR). In the first task of the research, perchlorate-reducing isolates were cultured; this effort was complemented with the utilization of a culture-independent method (T-RFLP) to more fully characterize the microbial community present in a fixed-bed (FXB) reactor treating perchlorate-contaminated water at a field site. The next two tasks were focused on assessing the potential for the Prokaryotic cDNA Subtraction method to identify functional genes related to perchlorate reduction in an unsequenced, environmental PRB. In the final task, the quantity and transcription of genes related to perchlorate reduction were investigated.

The microbial community present in a pilot-scale FXB reactor for perchlorate reduction in Rialto, California was assessed by culture-dependent and culture-independent methods. T-RFLP, the culture-independent method, demonstrated the presence of a more diverse PRB community than was evident by culturing. In particular, the culturing technique indicated that *Dechlorospirillum* was the only PRB in the reactor, while T-RFLP found a more diverse range of PRB including *Dechloromonas* and other members of the *Rhodocyclaceae* family, as well as *Ideonella dechloratans* (a chlorate-reducing bacterium). While partial or complete sequences are

[1] Henceforth, Prokaryotic SSH PCR cDNA Subtraction will be referred to as Prokaryotic cDNA Subtraction.

available for functional genes related to perchlorate reduction (e.g., *pcrA* and *cld*) in *Dechloromonas* and *Dechlorospirillum*, no such information is available for other members of the *Rhodocyclaceae* family and indeed for most environmental bacteria.

To expand the limited database of sequences related to perchlorate reduction, we applied Prokaryotic cDNA Subtraction to PRB strain JDS4. This effort marked the first time Prokaryotic cDNA Subtraction had been applied to a microorganism with an unsequenced genome. Using the same protocol that we had demonstrated previously using a model, fully sequenced microorganism (De Long et al., 2008), we applied this protocol to JDS4 degrading perchlorate. None of the genes identified were related to perchlorate reduction, and ninety-seven percent of the genes identified were rRNA genes. These results indicate that the current protocol for Prokaryotic cDNA Subtraction is insufficient to fully remove the residual rRNA from the purified mRNA, which is necessary to isolate genes related to perchlorate reduction. We are currently in discussions with Ambion, Inc. and Clontech Laboratories, as they have both expressed interest in helping us to improve the Prokaryotic cDNA Subtraction protocol.

The presence of the functional genes (i.e., DNA) responsible for perchlorate reduction is one line of evidence indicating that perchlorate reduction is possible at a given site; however, more compelling evidence of biological perchlorate reduction is the active transcription of the genes (i.e., RNA) associated with perchlorate reduction. We utilized existing primer sets that target *cld* and *pcrA*, and we demonstrated that these genes were present in the microbial community of the pilot-scale FXB reactor. We also utilized an enrichment culture developed from this field site to measure transcripts related to perchlorate reduction over a range of culturing conditions. To our knowledge, we are the first to have measured the transcription of genes related to perchlorate reduction. Our data show that active biological perchlorate reduction correlates to the expression of *cld*. While *cld* was strongly expressed under perchlorate-reducing conditions, it was not expressed under aerobic conditions and its expression was quickly curtailed under anoxic conditions. Thus, transcriptional data will be useful to gauge the activity of PRB in a process. Given the utility of this type of data, we are extending the *pcrA* qPCR assay of Nozawa-Inoue et al. (2008) to develop an RT-qPCR assay; we have also developed a qPCR assay for *cld* and will extend that to an RT-qPCR assay. We will apply these quantitative assays to our current bench-scale FXB reactors to demonstrate the correlation between perchlorate reduction and transcription of genes related to perchlorate reduction under different water quality conditions.

The combination of MBT used in this project allowed us to assess the composition of the microbial community during active perchlorate reduction as well as to detect the presence and expression of genes associated with perchlorate reduction. These MBT can be used to provide additional lines of evidence for biological perchlorate reduction, to gauge the activity of PRB in a process, and perhaps act as an early warning system for upsets to the PRB community.

Our transition plan is to build upon the results from this SEED project by demonstrating the utility of RT-qPCR assays targeting gene transcripts related to perchlorate reduction in our bench-scale FXB reactors and at a field site. In addition, we strongly believe in expanding the limited database of gene sequences related to perchlorate reduction, and we plan on modifying our Prokaryotic cDNA Subtraction protocol to render it successful for PRB.

1.0 Objectives

Molecular Biology Tools (MBT) have the potential to improve the design and operation of both *in situ* and *ex situ* biological treatment processes such as those targeting the reduction of perchlorate to innocuous chloride. The main objective of this study was to investigate the utility of MBT to improve our ability to monitor and understand biological perchlorate reduction. Detecting the presence of specific functional genes requires *a priori* knowledge of target gene sequences. In the case of biological perchlorate treatment, it remains unclear whether sufficient gene sequences are available to design primers for accurate quantification of the key genes involved in perchlorate reduction for all relevant PRB species. Therefore, the first task of this project was to characterize the microbial community in a fixed-bed (FXB) reactor treating perchlorate-contaminated water (pilot-scale biologically active carbon filters operated by Carollo Engineers in Rialto, California) by the culture-independent terminal restriction fragment length polymorphism (T-RFLP) technique; the purpose of this task was to determine if the PRB present were phylogenetically related to bacteria for which perchlorate reduction gene sequences are available. The second and third tasks of the project were to determine if a MBT called Prokaryotic cDNA Subtraction could be applied to an environmental PRB whose genome was not fully sequenced; the purpose of this task was to obtain gene sequences related to perchlorate reduction from a PRB that has not been well-characterized. The fourth task of the project was to develop and/or apply quantitative PCR (qPCR) assays targeting genes related to perchlorate reduction or reverse-transcription PCR (RT-PCR) assays targeting gene transcripts related to perchlorate reduction; the purpose of this task was to investigate if these assays can provide useful information about the perchlorate-reducing potential or activity under different environmental conditions.

This combination of MBT allowed us to assess the composition of the microbial community during active perchlorate reduction as well as to detect the presence and expression of genes associated with perchlorate reduction.

2.0 Background

Section 2.1 provides an overview of the types of MBT that are suitable for the study of biological processes and discusses the benefits and drawbacks of the various approaches. Since the application of MBT is predicated on *a priori* knowledge of the target gene sequence(s), Section 2.2 discusses methods for obtaining the requisite gene sequences. The microbiology of perchlorate reduction is reviewed in Section 2.3, and available biological perchlorate treatment technologies are summarized in Section 2.4.

2.1 Studying Biological Treatment with MBT

MBT are becoming increasingly popular for the interrogation of biological treatment processes (Sharkey et al., 2004; Rittmann, 2002). The overwhelming majority of applications have sought to determine the phylogenetic identity of the species present, in part due to the ease of assigning phylogeny via molecular methods. To identify phylogenetic groups of bacteria, molecular tools target the 16S (small subunit) ribosomal ribonucleic acid (rRNA) gene. For example, fluorescence *in situ* hybridization (FISH) uses fluorescently labeled nucleic acid probes targeting rRNA molecules to locate and quantify bacteria with specificity at the genus or species level (Amann et al. 2001; 1995). This technique has been applied to microbial communities treating contaminated water or wastewater (Beer et al., 2002; Silyn-Roberts and Lewis, 2001; Stoffels et al., 1998; Kämpfer et al., 1996). Gene microarrays for 16S rRNA have been designed to simultaneously assay for the presence of many different bacterial species (Koizumi et al., 2002; Small et al., 2001; Guschin et al., 1997). Terminal restriction fragment length polymorphism (T-RFLP) is a microbial community fingerprinting technique that often targets the rRNA gene; it has been applied to describe the microbial communities in water (Rahm et al., 2006; Regan et al., 2002), soil (Chim Chan et al., 2008), and sediments (Kittelmann and Friedrich, 2008). Quantitative PCR (qPCR) is popular for quantifying bacteria because of the low detection limit. For instance, qPCR has been used to quantify *Nitrospira* spp. in activated sludge (Hall et al., 2002).

However, solely determining the phylogenetic identity of the bacteria that populate a system has limited utility because phylogeny is not necessarily predictive of metabolic capability (Jaspers and Overmann, 2004); this is certainly the case for PRB because of their phylogenetic diversity (Coates et al., 1999). Targeting functional genes (i.e., genes that encode biodegradation enzymes) offers more insight into fundamental biological processes. A number of researchers have successfully used MBT targeting functional genes for environmental applications. For instance, Wu et al. (2001) generated a functional gene array containing probe spots for nitrite reductase (*nirS* and *nirK*), ammonia monooxygenase (*amoA*), and methane mono-oxygenase (*pmoA*) from a variety of different bacterial strains. qPCR has been used to detect genes associated with degradation of organics such as toluene and xylene (Baldwin et al., 2003; Beller et al., 2002). Cole et al. (2004) used qPCR to detect genes required for ammonia oxidation (*amoA*) and denitrification (*nirS* and *nirK*) in membrane-aerated biofilms. Recently, a qPCR assay has been developed to quantify copies of *pcrA*, which is a gene encoding a subunit of the perchlorate reductase enzyme (Nozawa-Inoue et al., 2008).

The studies mentioned in the previous paragraph targeted DNA, which indicates functional *potential* but not *activity*. Metabolic reactions cannot occur unless the key genes are expressed, and gene expression often is regulated in response to environmental factors. Gene transcripts (i.e., messenger RNA [mRNA]) represent the expressed genes, and, therefore, quantifying gene transcripts is a better predictor of activity. In some cases, post-transcriptional regulation may not lead to an active enzyme; however, the presence of gene transcripts generally indicates production of the encoded enzyme. Some researchers have detected gene transcripts in environmental samples and laboratory cultures degrading contaminants. Reverse-transcription polymerase chain reaction (RT-PCR) has been used to detect functional gene expression in naphthalene-contaminated groundwater (Wilson et al., 1999) and chlorobenzene-contaminated soil (Alfreider et al., 2003). RT-qPCR (reverse-transcription quantitative PCR) has been employed to quantify gene transcripts for degradation of 1,2,4-trichlorobenzene in river sediments (Meckenstock et al., 1998). Johnson et al. (2005a; 2005b) and Lee et al. (2006) used RT-qPCR to determine the number of copies of trichloroethene reductive dehalogenase gene transcripts in semi-batch reactors and showed that expression of the reductive dehalogenase genes correlated with the desired dechlorination of trichloroethene beyond cis-dichloroethene.

To develop a clear picture of the metabolic potential and current activity of a given microbial community, it might be desirable to quantify 16S rRNA genes, functional genes (DNA), and mRNA simultaneously. Pernthaler and Amann (2004) developed a method for simultaneously using rRNA-FlSH and mRNA-FISH to determine the identity and activity of bacteria *in situ*. qPCR also has been used to monitor 16S rRNA genes and an ammonia oxygenase gene in wastewater treatment reactors (Layton et al., 2005; Harms et al., 2003; Dionisi et al., 2002). To compare metabolic potential with activity, Qiu et al. (2004) monitored genomic copies and mRNA transcripts of a copper-dependent nitrite reductase (*nirK*) using qPCR. They found that environmental samples containing more genomic copies of the *nirK* gene did not show higher levels of *nirK* gene transcripts, indicating that monitoring DNA copies of a functional gene is not necessarily a good predictor of gene transcription. Recently, qPCR and RT-qPCR have been used to quantify levels of 16S rRNA genes for *Dehalococcoides* and reductive dehalogenase genes and gene transcripts at trichloroethylene-contaminated sites (Lee et al., 2008).

2.2 Methods for Obtaining Gene Sequences for Biodegradation Genes

MBT have the potential to improve the design and operation of biological treatment processes. However, the application of many MBT is predicated on *a priori* knowledge of target nucleic acid sequences, and the current dearth of such biomarkers represents a fundamental barrier to the routine application of MBT at DoD field sites (Strategic Environmental Research and Development Program [SERDP] and Environmental Security Technology Certification Program [ESTCP], 2005). A critical need, therefore, exists for tools that can rapidly identify the functional target genes.

Historically, obtaining sequences of environmentally relevant functional genes has been a tedious process. A wide variety of approaches have been employed. Many techniques involve direct selection of desired genomic library clones or screening genomic library clones (Brown, 2001). These methods involve screening hundreds of colonies to identify those that contain the

gene(s) of interest. Another approach is to use primers based on a similar gene to amplify the gene of interest in a different microorganism (Bender et al., 2004). This approach is commonly applied to obtain environmentally relevant functional gene sequences; however, it is predicated on the *a priori* sequencing of a similar gene and cannot be applied to genes with novel functions or genes with analogous function but distinct gene sequences.

With the advent of automated DNA sequencing, it is possible to sequence whole genomes. For organisms with fully sequenced genomes, researchers can begin with gene sequences and subsequently assign functions. Newly obtained gene sequences are typically compared to previously obtained gene sequences, and sequence similarities are used to infer gene function; however, only 60% of newly sequenced genes can be assigned a function using this approach (Wackett and Hershberger, 2001). For genes with novel functions, other methods must be used.

Methods based on differential gene expression are attractive because they can isolate genes associated with a particular function. Microarrays, differential display, RNA arbitrarily primed PCR (RAP-PCR), and representational difference analysis (RDA) have been used to identify functional genes (Bathe and Norris, 2007; Domínguez-Cuevas et al., 2006; Paulino et al., 2002; Becker et al., 2001; Shepard and Gilmore, 1999; Rivera-Marrero et al., 1998; Fleming et al., 1998). Microarrays are powerful tools for identifying differentially expressed genes, but the design of a new microarray requires significant investment. Differential display, RAP-PCR, and RDA often have high false-positive rates and might miss some differentially expressed genes due to PCR biases (Paulino et al., 2002; Nagel et al., 2001; Shepard and Gilmore, 1999; Bowler et al., 1999; Rivera-Marrero et al., 1998; Fleming et al., 1998). RDA uses multiple high-stringency hybridizations that could result in loss of low-abundance genes (Bathe and Norris, 2007; Becker et al., 2001). We have recently demonstrated that Prokaryotic cDNA Subtraction can be used to identify differentially expressed functional genes in a model prokaryotic system (De Long et al., 2008). This technique, described in detail in Section 2.2.1, was used in the current project.

2.2.1 Prokaryotic cDNA Subtraction

cDNA Subtraction is an attractive method for the isolation of environmentally relevant genes because it amplifies only those genes that are specifically expressed under one chosen set of conditions (e.g., perchlorate-reducing conditions). cDNA Subtraction had previously been developed for application to eukaryotes (Sugimoto et al., 2004; Zhang et al., 2001; Diatchenko et al., 1996; Plum et al., 1994; Kinger and Tyagi, 1993) due to the ease of isolating eukaryotic mRNA through its polyA tail. We are the first group to have successfully extended the technique for application to prokaryotes (De Long et al., 2008), whose mRNA lacks this polyA tail.

The Prokaryotic cDNA Subtraction method is summarized as follows (Figure 2.1). Bacteria are grown in the presence and absence of a pollutant (tester and driver cultures, respectively); mRNA is isolated from both cultures and used to synthesize double-stranded cDNA. The cDNA is digested to prepare the ends for adaptor ligation. One aliquot of tester cDNA is ligated to adaptor 1, and the other aliquot is ligated to adaptor 2 (Figure 2.1, step I). After heat denaturing, the tester cDNA pools are hybridized to an excess of heat-denatured driver cDNA (Figure 2.1, step II). These two pools are mixed together, and additional heat-denatured driver cDNA is

added in a second hybridization; DNA polymerase is used to fill in the overhang ends (Figure 2.1, step III). cDNA fragments in the tester 1 pool that were single-stranded after the first hybridization can now hybridize to their complements in the tester 2 pool. This produces a pool of double-stranded cDNA fragments representing the differentially expressed genes, which have adaptor 1 and 2 on opposite ends (type a in Figure 2.1, step III); these genes are selectively amplified in suppression PCR through the use of primers that are complementary to the adaptors (Figure 2.1, step IV). Those fragments with no adaptors or adaptors only on one end (types b, e, f, and g in Figure 2.1, step III) do not have two primer binding sites and cannot be amplified.

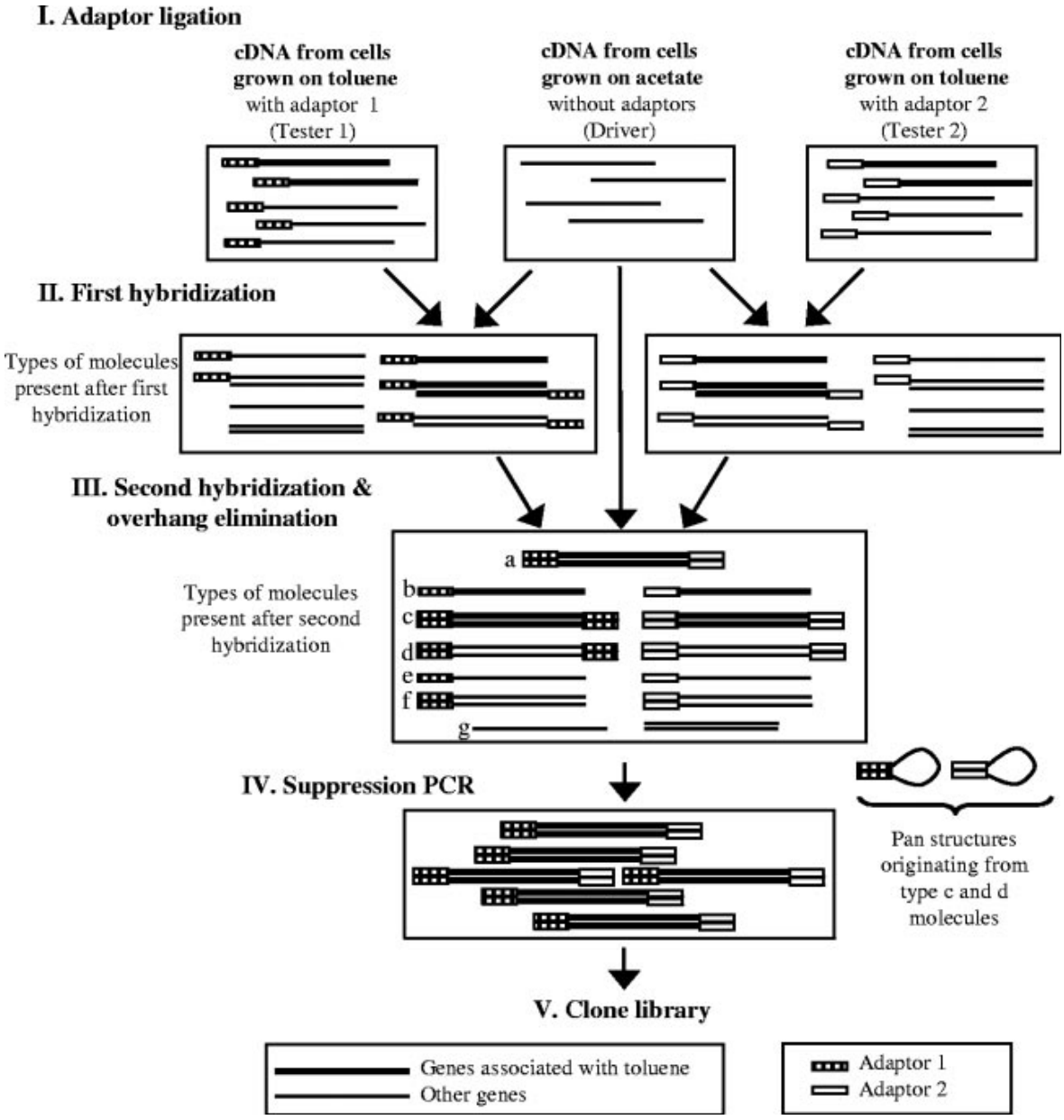

Figure 2.1. Schematic of Prokaryotic cDNA Subtraction.
(Reproduced from De Long et al. (2008) with permission from the publisher)

(Reproduced from De Long et al. (2008) with permission from the publisher)
Amplification of molecules containing the same adaptor on both ends (types c and d in Figure 2.1, step III) is suppressed because of hybridization between the adaptor on the 5 foot end and its complement on the 3 foot end. During the first hybridization, abundant genes form more hybrids with the same adaptor on both ends as compared to rare genes. Suppressing the amplification of these molecules increases the diversity of the subtracted gene pool. The amplicon from suppression PCR is cloned (Figure 2.1, step V) and sequenced.

We extended the cDNA Subtraction method from eukaryotes to prokaryotes, and applied it to the model system of *Pseudomonas putida* mt-2 (which has a fully sequenced genome) degrading toluene (De Long et al., 2008). Twenty-one of the isolated gene fragments generated by Prokaryotic cDNA Subtraction were sequenced. Ninety-five percent of these gene sequences were related to toluene degradation even though these genes represent less than 0.2% of the 5500 genes in the genome (Greated et al., 2002; Nelson et al., 2002). The detected sequences represent 10 distinct genes within two key toluene biodegradation operons. Although only a small number of the gene fragments isolated by Prokaryotic cDNA Subtraction were sequenced, we obtained sequences for 50% of the genes involved in toluene degradation. These data demonstrate that Prokaryotic cDNA Subtraction was successful at identifying key genes related to toluene degradation. Any of these genes could potentially serve as biomarkers for toluene degradation *in situ*.

2.3 Microbial Perchlorate Reduction

Many PRB have been isolated from activated sludge, anaerobic digesters, and environmental samples (Balk et al., 2008; Shrout et al., 2006; 2005; Waller et al., 2004; Bruce et al., 1999; Coates et al., 1999; Rikken et al., 1996; Wallace et al., 1996). They appear to be ubiquitous in the environment (Coates et al., 1999), making it possible to use indigenous bacteria in treatment scenarios. PRB have been identified among a broad range of phylogenetic groups including the alpha, beta, gamma, and epsilon subclasses of the *Proteobacteria* (Coates et al., 1999). Perchlorate is reduced to chlorate and chlorite by perchlorate reductase (encoded by the *pcrABCD* genes) (Bender et al., 2005). Chlorite is a toxic byproduct that is reduced to chloride by chlorite dismutase (encoded by the *cld* gene).

PRB can use a wide variety of electron donors including many simple organics (e.g., acetate), hydrogen, and even Fe (II) (Shrout et al., 2005; Lack et al., 2002; Bruce et al., 1999; Coates et al., 1999). They are facultative aerobes and can use a wide array of electron acceptors in addition to perchlorate, including oxygen, nitrate, and Mn (IV) (Bruce et al., 1999; Coates et al., 1999; Kengen et al., 1999). Oxygen and nitrate are generally preferred and are typically utilized first; however some strains appear to simultaneously reduce multiple electron acceptors (Nozawa-Inoue et al., 2005; Zhang et al., 2005; Chaudhuri et al., 2002). The trace metal molybdate is required for perchlorate reduction because it is a cofactor for perchlorate reductase.

Little is known about how PRB behave at perchlorate-contaminated sites and in bioreactors treating perchlorate-contaminated water. Zhang et al. (2005) used ribosomal intergenic spacer analysis (RISA) to track the PRB strain *Dechlorosoma* KJ in a perchlorate-treatment bioreactor

that was initially inoculated with this strain. However, after 6 months of operation this strain could no longer be detected while other strains of PRB (*Dechloromonas*) were apparent. To specifically and simultaneously track all PRB, MBT designed to target functional genes related to perchlorate reduction (*pcrABCD* and *cld*) are more desirable, especially given the phylogenetic diversity of PRB. qPCR is a popular tool for quantification, but target gene sequences are required for primer design. The complete gene sequences encoding perchlorate reductase (*pcrABCD*) and chlorite dismutase (*cld*) are known for two strains of PRB (Table 2.1). For a number of other strains, only partial gene sequences related to perchlorate reduction are available, and no sequences related to perchlorate reduction are available for others (Nozawa-Inoue et al., 2008; Bender et al., 2005; Bender et al., 2002; Coates et al., 1999).

Table 2.1 Gene Sequences Related to Perchlorate Reduction.

Selected Strains	*cld* Gene Sequence *	*pcr* Gene Sequence **
Dechloromonas aromatica	Complete genome sequence	
Dechloromonas agitata	Complete	Complete
Azospira sp.	Partial	None
Dechloromarinus sp.	Partial	None
Dechlorospirillum sp.	Partial	None
Pseudomonas PK	Partial	None
Azospirillum PRB	None	None
Moorella perchloratireducens	None	None
Strain JDS4	None	None
Wolinella succinogenes HAP-1	None	None

The *Dechloromonas aromatica* genome is available online (http://www.ncbi.nlm.nih.gov/).
* Bender et al., 2004; 2002
** Nozawa-Inoue et al., 2008; Bender et al., 2005

PCR primers have been reported in the literature for both the *pcrA* and *cld* genes based on the limited number of known sequences. Bender et al. (2004) developed a set of nested PCR primers targeting the *cld* gene and successfully detected these genes in laboratory cultures and environmental samples. Nozawa-Inoue et al. (2008) developed a set of primers targeting the *pcrA* gene, which is the catalytic subunit of perchlorate reductase, and detected this gene in pure cultures and soil samples. Additionally Nozawa-Inoue et al. (2008) developed *E. coli* plasmids containing the cloned *pcrA* gene from *Dechloromonas agitata* strain CKB to be used as a gene copy number standard for quantifying *pcrA* genes via qPCR. Some bacteria possess *pcr* genes but not a *cld* gene, which is required for chlorite reduction. These bacteria cannot survive in the presence of high perchlorate because chlorite will build up to toxic levels (Bender et al., 2004). Therefore, monitoring *pcr* genes alone is not a clear predictor of perchlorate-reducing capability. Furthermore, other bacteria such as chlorate-reducing bacteria contain the *cld* gene but cannot degrade perchlorate, suggesting that *cld* cannot be monitored alone either (Nozawa-Inoue et al., 2008; Stenklo et al., 2001). Therefore, it is desirable to monitor both the *pcr* and *cld* genes to get a clear picture of PRB activity. No qPCR protocols are currently available for the *cld* gene.

2.4 Biological Perchlorate Treatment

Perchlorate is a widespread groundwater contaminant (Renner, 1998). It is a highly soluble and stable contaminant (Urbansky and Brown, 2003), and it is known to cause adverse human health effects including disruption of thyroid function (Wolff, 1998). In addition to groundwater and drinking water, perchlorate has been detected in dairy milk and breast milk (Kirk et al., 2005). To protect public health, the United States Environmental Protection Agency (USEPA) has established an official reference dose of 0.0007 mg/kg body weight/ day, which corresponds to a drinking water standard of 24.5 µg/L (USEPA, 2006). The California Department of Health Services has set the maximum contaminant level even lower at 6 µg/L (California Department of Health Services, 2007).

Due to the prevalence of perchlorate contamination, reliable and cost effective *in situ* and *ex situ* treatment methods are needed. The two main treatment options that have proven effective are ion exchange and biological reduction. Ion exchange generates waste brine and becomes very expensive for high perchlorate concentrations. Biological treatment is a promising approach and transforms perchlorate to innocuous chloride (Hatzinger, 2005). Bacteria use perchlorate as the electron acceptor, degrading perchlorate under anaerobic conditions. An energy source (e.g., acetate or hydrogen) must be provided. A number of different bioreactor configurations have been evaluated and some have been demonstrated at the pilot- and full-scale (Sutton, 2006). In laboratory- and pilot-scale reactors, perchlorate has been treated *ex situ* using fluidized bed reactors, FXBs, and moving-bed biofilm reactors; only fluidized bed reactors have been used at full-scale for treatment of perchlorate-contaminated groundwater (Sutton, 2006; Wallace et al., 1998). With funding from ESTCP, FXBs have recently been tested and evaluated for application to drinking water treatment (ER-0544: Direct Fixed-Bed Biological Perchlorate Destruction Demonstration, Carollo Engineers). Laboratory studies have shown this to be a viable approach (Choi et al., 2007; Min et al., 2004; Brown et al., 2003; 2002; Logan and LaPoint, 2002; Kim and Logan, 2001; 2000; Logan, 2001; Giblin et al., 2000a; 2000b; Miller and Logan, 2000; Wallace et al., 1998).

Effective reactor design must consider the source and ultimate use of the perchlorate-contaminated water, the influent perchlorate concentration, and the concentration of competing electron acceptors (i.e., oxygen and nitrate). A suitable energy source must be provided to reduce all or nearly all competing electron acceptors and perchlorate. For drinking water treatment, oxygen and nitrate concentrations typically drive the required concentration of electron donor because they are present in much higher concentrations (mg/L), and perchlorate is typically present at low levels (µg/L). A number of different organic electron donors have been used successfully including acetate, ethanol, lactate, and pyruvate (Choi et al., 2007; Min et al., 2004; Brown et al., 2003; 2002; Kim and Logan 2001; 2000; Logan, 2001; Giblin et al., 2000a), and the inorganic electron donor hydrogen has also been shown to support biological perchlorate reduction (Logan and LaPoint, 2002; Logan, 2001; Giblin et al., 2000b).

Although the feasibility of biological perchlorate treatment has been demonstrated, barriers still exist (e.g., the risk of process upsets and failures) that prevent its widespread application. FXB reactors require periodic backwashing to remove biomass and prevent filter clogging. Upsets can

be caused by over-backwashing (Choi et al., 2007), or by changes in influent water characteristics or disruptions to the primary substrate feed. More work is needed to understand how microbial communities respond to varying influent water characteristics and operational changes. Furthermore, MBT are needed to track the microbial communities in biological perchlorate treatment processes during routine operation and after process upsets to ensure that there are no long-term negative impacts on the perchlorate-reducing microbial community. These tools would reduce the risks associated with the application of biological perchlorate treatment.

3.0 Materials and Methods

The objective of this project was to demonstrate the utility of MBT to improve our ability to monitor and understand biological perchlorate reduction. Four tasks, described as follows, were conducted to address this objective.

3.1. Task 1. Culture PRB and Characterize Mixed Microbial Community

The purpose of this task was threefold. First, we isolated or assembled a variety of pure cultures of PRB so that a novel PRB could be used for the Prokaryotic cDNA Subtraction in Task 2. Pure cultures were isolated from a pilot-scale FXB reactor treating perchlorate. Other pure cultures were obtained from collaborators (University of Toronto and University of Notre Dame). Second, we characterized the microbial community in the pilot-scale FXB reactor treating perchlorate and in a perchlorate-reducing enrichment culture to determine what phylogenetic groups of bacteria and functional genes involved in perchlorate reduction were present. This analysis was done to determine if the known PRB in these samples were species for which functional gene sequences related to perchlorate reduction are already available (e.g., *Dechloromonas aromatica, Dechloromonas agitata*). If gene sequences related to perchlorate reduction were not available for the types of PRB present in the samples, this would underscore the need to expand the functional gene database related to perchlorate reduction. The primers used to detect genes or gene transcripts related to perchlorate reduction will only be as good as the depth of the gene sequence database used to design them. Third, the PCR primers targeting perchlorate-related genes that were designed based on available sequences (Nozawa-Inoue et al., 2008; Bender et al., 2004) were tested to determine if some of the genes related to perchlorate reduction could be detected in the pure cultures or mixed microbial communities.

3.1.1 Materials and Methods for Task 1

Enrichment Culture. Samples were collected from the influent end of the pilot-scale FXB reactor (operated by Carollo Engineers in Rialto, California as part of ESTCP project ER-0544: Direct Fixed-Bed Biological Perchlorate Destruction Demonstration) during active perchlorate reduction. These samples were used to develop an enrichment culture by inoculating an anaerobic minimal environmental medium containing 5 mg/L of perchlorate. Perchlorate degradation was verified by ion chromatography. The concentration of perchlorate was measured via USEPA method 314 using a Dionex DX-600 ion chromatograph equipped with an AS-16 column, potassium hydroxide eluant, and 1000 μL injection loop.

Pure Cultures. Serial dilutions of the enrichment culture were plated on agar plates composed of minimal environmental medium containing 100 mg/L of perchlorate. The plates were incubated anaerobically at 30°C. Single colonies were selected and struck to purity twice. The isolates were screened for their ability to degrade perchlorate in batch culture using a perchlorate selective electrode (Thermo Electron Corporation, Beverly, Massachusetts). DNA was isolated from each perchlorate-degrading culture, and individual strains were phylogenetically identified by sequencing the 16S rRNA gene.

Collaborators from the University of Toronto (Waller et al., 2004) shipped several perchlorate-reducing *Azospirillum* strains, and collaborators from the University of Notre Dame (Shrout et al., 2006) shipped several perchlorate-reducing strains. The strains were struck on anaerobic environmental medium containing 100 mg/L perchlorate, and incubated at 30^{o}C under anaerobic conditions. DNA was isolated from each perchlorate-degrading culture, and individual strains were phylogenetically identified by sequencing the 16S rRNA gene.

Phylogenetic Characterization of Mixed Microbial Communities. Activated carbon samples from the pilot-scale FXB reactor (Rialto, California) were sonicated in a phosphate buffer to release cells from the activated carbon. The buffer was transferred to a separate tube, and cells were collected by centrifugation. For the FXB reactor samples and the enrichment culture, DNA was isolated using a kit (UltraClean™ Soil DNA Isolation Kit, Mo Bio Laboratories; Carlsbad, California). T-RFLP was conducted according to previously developed protocols (Egert and Friedrich, 2005; Marsh, 1999). Briefly, the 16S rRNA gene was amplified using a set of PCR primers that can amplify all Bacteria (8F and 1492R); the forward primer was fluorescently labeled with FAM. Enrichment culture DNA samples were PCR-amplified for 16 cycles. DNA samples from the FXB reactor were first PCR-amplified for 16 cycles, and amplicon from the first PCR reaction was amplified for an additional 25 cycles to produce sufficient amplicon for T-RFLP analysis. For all samples, two replicate PCR reactions were combined and subjected to post-amplification treatment with Klenow to fill in partially single-stranded amplicon (Egert and Friedrich, 2005). The PCR amplicon was digested with one of three restriction enzymes: HhaI, MspI, or RsaI and then desalted. Restriction fragments were separated by size on an ABI 3130 DNA analyzer. Individual T-RFs were putatively identified using the T-RFLP Analysis Program (Marsh et al., 2000).

Functional Gene Characterization. DNA was isolated from the FXB reactor samples, the enrichment culture, and each of the PRB strains isolated from the enrichment culture using a kit (UltraClean™ Soil DNA Isolation Kit, Mo Bio Laboratories; Carlsbad, California). PCR reactions were run to assay for the *pcrA* and *cld* genes using previously described primers and thermocycling parameters (Nozawa-Inoue et al., 2008; Bender et al., 2004). For *cld* gene detection, samples were first PCR-amplified using DCD-F and DCD-R primers; then amplicon from the first reaction was PCR-amplified by a nested PCR reaction using UCD-238F and UCD-646R primers as described previously by Bender et al. (2004). PCR amplicon was electrophoresed on an agarose gel to identify samples that yielded a PCR product of the appropriate size.

3.2 Task 2. Perform Prokaryotic cDNA Subtraction on a PRB

We published the first demonstration of Prokaryotic cDNA Subtraction (De Long et al., 2008), and validated the technique using a model system (i.e., *Pseudomonas putida* degrading toluene). The model system was chosen such that the target organism had a completely sequenced genome, and the genes involved in toluene degradation had already been well-delineated. Since Prokaryotic cDNA Subtraction had only been applied to a prokaryote with a fully sequenced genome, the purpose of this task was to apply Prokaryotic cDNA Subtraction to a PRB whose genome had not been fully sequenced. Thus, Prokaryotic cDNA Subtraction had the potential to

identify previously unknown genes involved in perchlorate reduction. Based on the results of Task 1, strain JDS4 (Shrout et al., 2006) was the PRB chosen for this task.

3.2.1 Materials and Methods for Task 2

Bacterial Strain and Culturing Conditions. Batch cultures of JDS4 were cultivated anaerobically in sealed serum bottles containing environmental medium with 100 mg/L of perchlorate. After medium addition, the bottles were purged with nitrogen gas to produce anaerobic conditions, sealed, and autoclaved. Samples were taken aseptically with syringe needles, and sterile nitrogen gas was injected to replace the liquid volume removed to maintain anaerobic conditions. For cultures that were converted to aerobic conditions, each batch culture was transferred to a sterile erlenmeyer flask and shaken.

Prokaryotic cDNA Subtraction. The Prokaryotic cDNA Subtraction procedure was applied to strain JDS4. As described above, batch cultures of JDS4 were grown under anaerobic conditions, and then half of the reactors were converted to aerobic conditions (tester and driver, respectively). Subsequent steps of the Prokaryotic cDNA Subtraction technique were carried out as described in De Long et al. (2008). One hundred and fifty-five clones from the cDNA Subtraction clone library were selected for screening to identify target 1,2-clones (i.e., those clones have 2 different adaptors at the ends). Thirty clones met the screen criteria and were selected for sequencing. Plasmids were isolated from selected clones using the FastPlasmid™ Mini Kit (Eppendorf; Westbury, New York). Clone inserts were sequenced at the University of Texas at Austin DNA Sequencing facility using M13 forward and reverse primers. The sequences were compared to publicly available sequences using the blastn algorithm (www.ncbi.mlm.nih.gov/BLAST).

3.3 Task 3. Assess Effectiveness of Current Prokaryotic cDNA Subtraction Protocol for Environmental Strains of PRB

The Prokaryotic cDNA Subtraction technique has been demonstrated to be successful in one prokaryotic organism (*P. putida*) where the target genes were very highly up-regulated (De Long et al., 2008). The purpose of this task was to examine the results from Task 2 to determine if the current Prokaryotic cDNA Subtraction protocol was sufficient for isolation of genes related to perchlorate reduction in environmental strains of PRB. If the current protocol had been sufficient, gene walking would have been carried out on the gene fragments sequenced in Task 2 to assemble whole gene sequences, and RT-qPCR would have been used to verify the up-regulation of these genes during active perchlorate reduction. Since the current Prokaryotic cDNA Subtraction protocol was insufficient for the identification of up-regulated genes related to perchlorate reduction, ideas for improving the protocol were discussed.

3.3.1 Materials and Methods for Task 3

Several key steps of the Prokaryotic cDNA Subtraction protocol were examined to assess how to test their efficiency or improve their performance.

3.4 Task 4. Examine the Quantity and Transcription of Genes Related to Perchlorate Reduction

The purpose of this task was to develop or apply assays that can quantitatively or semi-quantitatively measure genes or gene transcripts related to perchlorate reduction (i.e., *pcrA* and *cld*). The assays targeting genes (i.e., DNA) are useful for measuring the biological potential for perchlorate reduction at a contaminated site or in a treatment reactor. The assays targeting gene transcripts (i.e., mRNA) are useful for measuring biological activity for perchlorate reduction at a contaminated site or in a treatment reactor. While a qPCR assay for *pcrA* exists in the literature (Nozawa-Inoue et al., 2008), no qPCR assay exists for *cld*. Furthermore, the literature does not describe the transcription of genes related to perchlorate reduction under different environmental conditions. This task addressed those deficiencies.

3.4.1 Materials and Methods for Task 4

Culturing. Batch enrichment cultures were inoculated from the enrichment culture developed in Task 1. These cultures were cultivated anaerobically in sealed serum bottles containing environmental medium with 100 mg/L of perchlorate. After medium addition, the bottles were purged with nitrogen gas to produce anaerobic conditions, sealed, and autoclaved. Samples were taken aseptically with syringe needles, and sterile nitrogen gas was injected to replace the liquid volume removed to maintain anaerobic conditions. A fraction of these cultures was converted to aerobic conditions (after growth of sufficient biomass) by transferring the entire culture to a sterile erlenmeyer flask and incubating with shaking, while another fraction of the cultures was spiked with nitrate.

Biomass samples were collected from these cultures under perchlorate-reducing and non-perchlorate-reducing conditions. Non-perchlorate-reducing conditions were generated by culture aeration or the addition of 100 mg/L of nitrate. Biomass samples were collected just before the addition of competing electron acceptor and 4 and 20 hours after addition (when perchlorate reduction had ceased).

DNA and RNA extraction. Biomass samples were placed into Lysing Matrix E tubes (MP Biomedicals; Solon, Ohio) containing cetyl trimethylammonium bromide (CTAB) lysis buffer. Cells were lysed in a FastPrep 24 homogenizer (MP Biomedicals; Solon, Ohio). Lysis buffer was decanted into two clean DNase/RNase-free tubes. DNA was isolated with a kit (UltraClean™ Soil DNA Isolation Kit, Mo Bio Laboratories; Carlsbad, California); contaminating RNA was removed by RNase digestion. RNA was isolated using the RiboPure™- Bacteria kit (Ambion; Austin, Texas); contaminating DNA was removed by DNase digestion.

Copy Number Standard. The *pcrA* copy number standard was obtained from the laboratory of Professor Kate Scow, where this plasmid was developed previously (Nozawa-Inoue et al., 2008).

qPCR. Quantities of *pcrA* and *cld* were determined by qPCR using an ABI 7900HT Real-Time PCR System (Applied Biosystems; Foster City, California). To amplify the *pcrA* gene, previously described qPCR primers (pcrA320F and pcrA598R) were used (Nozawa-Inoue et al.,

2008) at a concentration of 0.2 μM. The following thermocycler program was used: 50°C for 2 min; 95°C for 10 min; 40 cycles: 95°C for 15 seconds and 60°C for 60 seconds. To amplify the *cld* gene, previously described end-point PCR primers (UCD-238F and UCD-646R) were used (Bender et al., 2004) at a concentration of 0.4 μM. The following thermocycler program was used: 50˚C for 2 min; 95°C for 10 min; 40 cycles: 95˚C for 15 seconds, 50°C for 60 seconds, and 60°C for 60 seconds. Amplicon was detected real-time using SYBR green nucleic acid stain (Power SYBR Green Master Mix, Applied Biosystems, Foster City, California). qPCR product dissociation curves were used to verify the specificity of the amplified product. *pcrA* gene copy number was determined from the C_T (copy number at which fluorescence crosses a selected threshold that occurs during the exponential phase of amplification) by comparison with the *pcrA* copy number standard. *cld* gene copy number was determined from the C_T by comparison with the *cld* copy number standard. Purified genomic DNA from *Dechloromonas agitata* was diluted and used as the copy number standard; the copy number was calculated based on an estimate of the size of the *D. agitata* genome and the fact that the *D. agitata* genome only contains one copy of the *cld* gene.

RT-PCR. To synthesize cDNA, 2 μg of DNase-treated total RNA were mixed with 2 μL of random hexamer primer (10 μM) and nuclease-free water (Ambion; Austin, Texas) to a final volume of 10 μL. The mixture was denatured at 70°C for 10 min and placed on ice. Four μL of First-Strand Buffer, 2 μL of DTT (0.1 M), 1 μL of dNTP mix (10 mM of each dNTP, New England Biolabs; Beverly, Massachusetts), 2 μL of water, and 2 μL of Superscript III (Invitrogen; Carlsbad, California) were mixed. The reactions were incubated at 25°C for 10 min and then 42°C for 1.5 hours. Negative controls, to verify the absence of contaminating genomic DNA, were prepared by omitting the reverse transcriptase. The following thermocycler program was used for PCR: 94°C for 2 min; 40 cycles: 94°C for 2 min, 50°C for one min, and 72°C for one min. Samples were collected after 20, 25, 30, 35, and 40 cycles. Amplicon was electrophoresed on a 1.5% agarose gel to visualize the quantity of amplicon for each time point and culturing condition.

4.0 Results and Accomplishments

The overall objective of this SEED project - to demonstrate the utility of MBT to improve our ability to monitor and understand biological perchlorate reduction – was met. As follows, this is described for each task.

4.1 Results from Task 1 (Culture PRB and Characterize Mixed Microbial Community)

4.1.1 Pure cultures. The *Azospirillum* PRB obtained from the University of Toronto were not able to be resuscitated presumably due to the thawing of the -80°C freezer in which they were stored at the University of Toronto. The PRB obtained from the University of Notre Dame were afflicted with a persistent bacterial contaminant, which took several months to separate from the PRB of interest. After purification, the 16S rRNA genes of these PRB were sequenced; strain JDS4 was determined to be a *Rhodocyclus* strain, making it phylogenetically distinct from the oft-studied *Dechloromonas* and *Dechlorosoma* PRB.

Thirteen perchlorate-reducing pure cultures were isolated from the enrichment culture that originally had been inoculated with microorganisms from the pilot-scale FXB reactor. All 13 isolates were determined to be *Dechlorospirillum* spp. by 16S rRNA gene phylogeny.

4.1.2 Phylogenetic Characterization of Mixed Microbial Community. As discussed above, the culturing of PRB from the enrichment culture yielded only *Dechlorospirillum* species. However T-RFLP, which is a culture-independent technique, provided evidence of a more diverse microbial community. Furthermore, the microbial communities in the two pilot-scale FXB reactors and the enrichment culture were highly similar (data not shown).

Figure 4.1 shows a representative T-RFLP electropherogram for the enrichment culture. Restriction fragments for the PRB *Dechloromonas* sp. and the chlorate-reducing bacteria *Ideonella dechloratans* were putatively identified. Peaks putatively identified as *Dechloromonas* also may correspond to members of the *Rhodocyclaceae* family, which includes some known PRB (Bender et al., 2002; Coates et al., 1999). A 16S rRNA gene clone library was generated for the FXB reactor samples in the laboratory of Professor Lutgarde Raskin (personal communication); T-RFLP putative identifications showed good agreement with clone library data with the exception that *Ideonella dechloratans* was not identified in the clone library. A number of other restriction fragments in the T-RFLP patterns might correspond to unknown PRB or non-perchlorate-reducing members of the community (e.g., *Zoogloea*). No known PRB outside the *Dechloromonas* and *Rhodocyclaceae* groups were identified, indicating that available primers for functional genes related to perchlorate reduction will detect at least some of the perchlorate-reduction genes present in this sample.

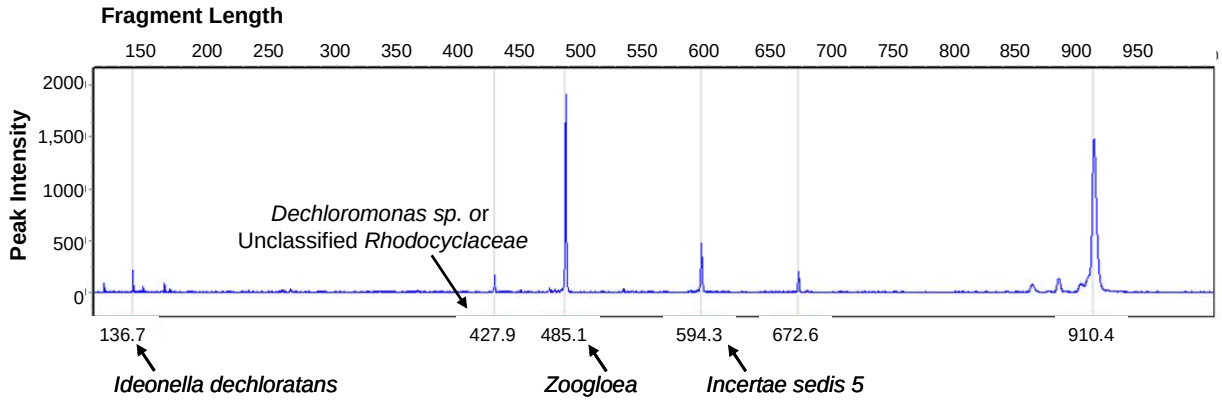

Figure 4.1 Representative Electropherogram for the Enrichment Culture. 16S amplicon was digested with MspI.

4.1.3 Functional Gene Characterization. *cld* and *pcrA* were detected in the DNA of all samples (Figure 4.2 and data not shown) using primers already available in the literature (Nozawa-Inoue et al., 2008; Bender et al., 2004). However, it is not possible to verify that all of the perchlorate-reducing genes in these samples were detected by these primers. This multi-pronged assessment (T-RFLP, *pcrA/cld* assays) suggests that MBT utilizing available primers targeting functional genes related to perchlorate reduction are likely to be suitable for interrogation of the pilot-scale FXB reactors treating perchlorate-contaminated water. However, few gene sequences related to perchlorate reduction are available for PRB in the *Rhodocyclaceae* family, outside of the *Dechloromonas* species. Thus, we chose to use isolate JDS4 (a *Rhodocyclus* strain) for Task 2, with the goal of obtaining sequences of genes involved in perchlorate reduction in a *Rhodocyclaceae* strain for which no functional gene sequences related to perchlorate reduction were currently available.

While these experiments interrogated for the presence of *cld*/*pcrA* in the DNA, it is much more important to address the transcription of functional genes related to perchlorate reduction because this more closely reflects the activity of PRB. This is addressed in Section 4.4.1.

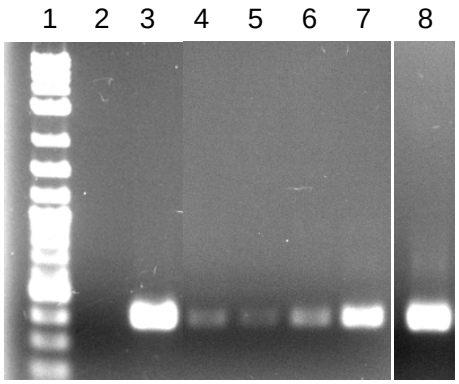

Figure 4.2 Gel of *cld* Genes Detected. Lanes (1): DNA size marker, (2) negative control, (3) positive control (*D. agitata*), (4)-(5) duplicate from FXB reactor 1, (6)-(7) duplicate from FXB reactor 2, (8) enrichment culture developed from FXB samples.

4.2 Results from Task 2 (Perform Prokaryotic cDNA Subtraction on a PRB)

The goal of Task 2 was to obtain gene sequences related to perchlorate reduction for *Rhodocyclus* PRB strain JDS4 (in the *Rhodocyclaceae*). This task represented the first attempt to apply Prokaryotic cDNA Subtraction, which is a method that can be used to isolate differentially expressed genes, to a microorganism without a fully sequenced genome. Strain JDS4 was selected for study because it was isolated from a perchlorate-contaminated site (Shrout et al., 2006), *Rhodocyclaceae* was a dominant group in the FXB reactors as shown by clone library data (Lutgarde Raskin, personal communication), and no functional gene sequences related to perchlorate reduction are available for *Rhodocyclus*. Since strain JDS4 had to be cultured anaerobically, a significant investment of time was required to procure the requisite mass of mRNA to perform the Prokaryotic cDNA Subtraction.

Of the 30 clones that were selected for sequencing after Prokaryotic cDNA Subtraction, four clone sequences were similar to the 16S rRNA gene, 25 clones sequences were similar to the 23S rRNA gene, and 1 clone sequence was similar to a subunit of an RNA polymerase. Thus, none of the clones contained fragments of genes related to perchlorate reduction (i.e., perchlorate reductase or chlorite dismutase genes).

4.3 Results from Task 3 (Assess Effectiveness of Current Prokaryotic cDNA Subtraction Protocol for Environmental Strains of PRB)

The results of Task 2 showed that the current protocol for Prokaryotic cDNA Subtraction is not sufficient to isolate genes related to perchlorate reduction in environmental PRB JDS4. This is likely due to the dominance of rRNA in the total RNA pool despite specific method steps designed to remove the majority of rRNA. Therefore, results to date indicate that method modifications are still needed to efficiently identify target biodegradation genes in environmental isolates.

It is possible that the rRNA removal method was not effective for strain JDS4 due to poor complementarity between the rRNA and the capture oligonucleotides that are supposed to bind and remove rRNA. However, after consultation with Ambion, Inc. (i.e., the company who makes the mRNA purification kit used in our protocol), we found that the capture oligonucleotides are a perfect match to the 16S rRNA gene of JDS4; despite the perfect match, 4 of the 30 clones sequenced contained a fragment of the 16S rRNA gene. In our work over the next few months, we plan to sequence the 23S rRNA gene of JDS4 and determine the complementarity between the capture oligonucleotides and the 23S rRNA gene. If the complementarity between the capture oligonucleotides and the 23S rRNA gene is not perfect, we will design a new capture oligonucleotide in conjunction with Ambion, Inc.

It is also possible that a higher rRNA removal efficiency might be required for environmental isolates than was required for the model system with which Prokaryotic cDNA Subtraction was validated (*P. putida* mt-2 degrading toluene). In the model system, the genes involved in toluene degradation are found on a plasmid, are extremely highly up-regulated (typically between 400- to 4,000-fold up-regulated under toluene-degrading conditions as compared to acetate-degrading conditions), and likely represent a large fraction of the total RNA pool as compared to

chromosomal genes. In contrast, the perchlorate-reducing genes are found on the chromosome and may be a much smaller fraction of the total RNA pool. Thus, even if the capture oligonucleotides are fully complementary to their rRNA targets, the current rRNA removal procedure may not be sufficient to detect the up-regulation of the perchlorate-related genes in our system. For example, rRNA constitutes around 95% of total rRNA; if 95% of the rRNA is removed during the mRNA purification, rRNA still constitutes 50% of the remaining RNA. Therefore, isolation of genes related to perchlorate reduction might require that rRNA removal efficiency be closer to 100%. For instance, three rounds of mRNA purification might be needed instead of the two rounds of mRNA purification that are currently used in the protocol. Another possible way to overcome the predominance of rRNA in the clone library would be to modify the driver used in the Prokaryotic cDNA Subtraction protocol. Currently, mRNA is purified for the driver culture and cDNA from this mRNA is hybridized in excess to the cDNA of the tester cultures. To increase the chance that 16S/23S cDNA from the driver will hybridize to 16S/23S cDNA from the tester cultures (which effectively prevents these molecules from being amplified in suppression PCR), the driver cDNA could be synthesized from total RNA instead of mRNA. Given the short-time frame for SEED projects, these modifications could not be attempted in the current project. In subsequent months, we plan to examine these modifications to the Prokaryotic cDNA Subtraction protocol and perform another application of Prokaryotic cDNA Subtraction to PRB JDS4. We are currently in discussions with Clontech Laboratories (Mountain View, California), who makes a kit for the eukaryotic version of cDNA Subtraction; they have expressed interest in contributing to our on-going work with Prokaryotic cDNA Subtraction (i.e., provision of reagents).

Since no new sequences related to perchlorate reduction were identified as a result of Tasks 2 and 3, Task 4 was completed using published primer sets that were based on already-available gene sequences related to perchlorate reduction (e.g., Nozawa-Inoue et al., 2008; Bender et al., 2004).

4.4 Results from Task 4 (Examine the Quantity and Transcription of Genes Related to Perchlorate Reduction)

4.4.1 Transcription of Genes Related to Perchlorate Reduction (RT-PCR)

Preliminary studies have shown that the *pcrA* and *cld* genes can be detected in the enrichment culture using previously described primers and endpoint PCR (see Section 4.1.3). To determine if *cld* gene expression correlates with perchlorate reduction, *cld* gene expression was measured by RT-PCR in JDS4 under perchlorate-reducing and non-perchlorate-reducing conditions. Three identical batch cultures of JDS4 were grown anaerobically under perchlorate-reducing conditions, and perchlorate degradation was verified using a perchlorate-selective electrode. Then, at time zero, one culture was aerated, the second culture was spiked with 100 mg/L nitrate, and the third was maintained under perchlorate-reducing conditions as a control. Over a period of 20 hours, biomass samples were collected, and perchlorate concentrations were measured. Within 4 hours of the addition of oxygen or nitrate, perchlorate degradation could not be detected using the perchlorate-selective electrode, while perchlorate continued to disappear in the control bottle. RNA was extracted from the biomass samples, cDNA was synthesized, and PCR reactions were run. *cld* transcripts were detected at all times in the control culture (Figure 4.3). In

the culture with nitrate, *cld* transcripts declined after 4 hours and were almost undetectable after 20 hours. In the culture with oxygen, the *cld* transcripts could not be detected after 4 hours. This demonstrates that for JDS4, perchlorate gene expression correlates with perchlorate-reducing activity. Additionally, these results indicate that the previously described primers and endpoint PCR thermocycler parameters can be used to detect gene transcripts for the *cld* genes.

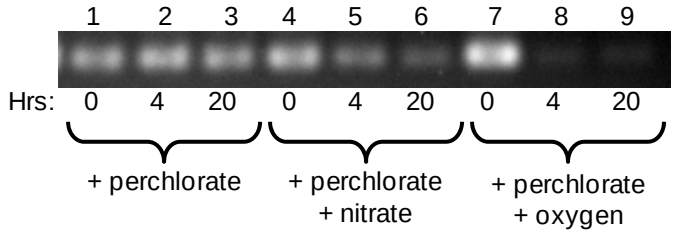

Figure 4.3. Gel of *cld* Gene Expression. All three cultures were grown with 100 mg/L of perchlorate and were actively degrading perchlorate at time zero hours. At time zero, one culture was maintained as a control culture, nitrate was added to a second culture, and a third culture was aerated. Lanes (1)–(3): control culture, (4)-(6) culture spiked with 100 mg/L nitrate at time zero, (7)-(9) culture aerated at time zero.

While the presence of genes related to perchlorate reduction has been measured by several research groups, to our knowledge, this is the first time that transcripts related to perchlorate reduction have been measured. This is extremely important because transcriptional data for functional genes related to perchlorate reduction can be used to monitor the health of an *in situ* or *ex situ* process. That is, to provide an additional line of evidence for biological perchlorate reduction and to gauge the activity of PRB in a process, we must interrogate the system at the mRNA level.

4.4.2 Quantification of Genes Related to Perchlorate Reduction (qPCR)

A qPCR assay has already been published for *pcrA* (Nozawa-Inoue et al., 2008), and we prepared a standard curve between C_T (copy number at which fluorescence crosses a selected threshold that occurs during the exponential phase of amplification) and the *pcrA* copy number standard (Figure 4.4). We are currently developing an RT-qPCR assay for *pcrA*; this will be tested on bench-scale FXB reactors for perchlorate-reduction under different nutrient conditions (with and without molybdate addition) that we have set up in our lab.

While an endpoint PCR assay has been published for *cld* (Bender et al., 2004), no qPCR assay exists for this gene. Using the same primers as the endpoint PCR assay, we developed a qPCR protocol for *cld*. We prepared a standard curve between C_T and the *cld* copy number standard (Figure 4.5). We are currently developing an RT-qPCR assay for *cld*; this will be tested on bench-scale FXB reactors for perchlorate-reduction under different nutrient conditions (with and without molybdate addition) that we have set up in our lab.

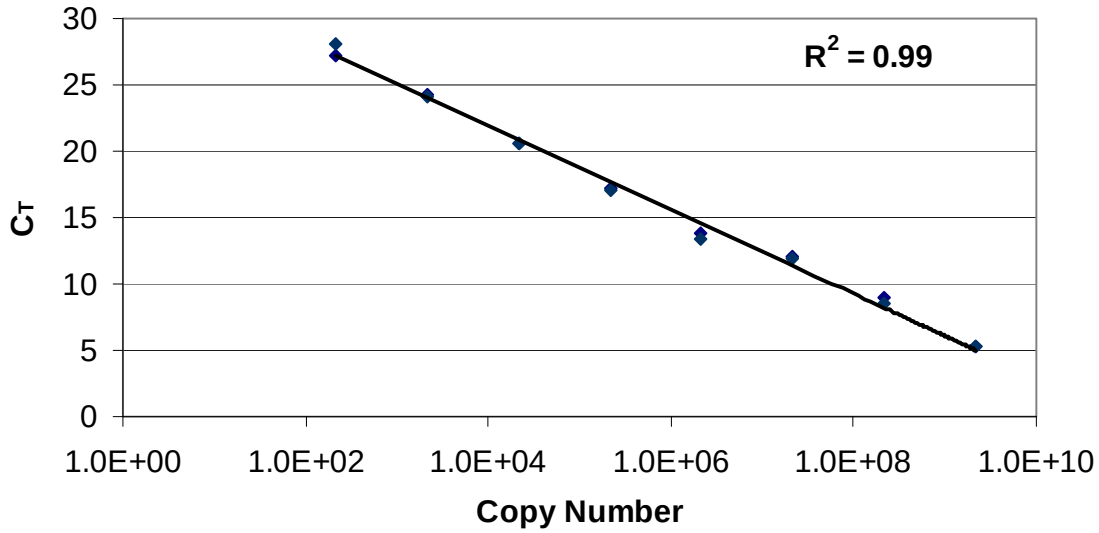

Figure 4.4. Standard Curve for the *pcrA* qPCR Assay. Purified plasmid containing the *pcrA* gene from *Dechloromonas agitata* was diluted for use as a copy number standard.

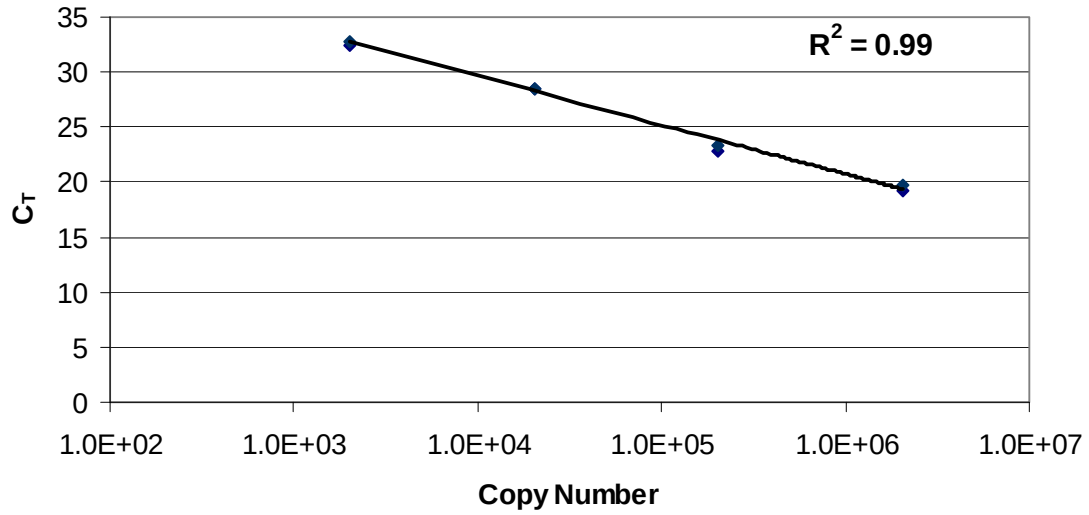

Figure 4.5. Standard Curve for the *cld* qPCR Assay. Purified genomic DNA from *Dechloromonas agitata* was diluted and used as a copy number standard. The copy number was calculated based on an estimate of the size of the *D. agitata* genome and the fact that the *D. agitata* genome only contains one copy of the *cld* gene.

As discussed in section 4.4.1, no assays to measure the transcription of genes related to perchlorate reduction currently exist, and these types of assays are necessary to provide an additional line of evidence for biological perchlorate reduction and to gauge the activity of PRB in a process. Quantitative assays targeting the mRNA, such as we are developing for *pcrA* and *cld*, could be used as a warning system to indicate an upset in the PRB microbial community.

5.0 Conclusions

Ammonium perchlorate, a solid rocket fuel oxidant, has contributed to groundwater contamination in the U.S. Fortunately, perchlorate can be biologically reduced to innocuous chloride. In this work, we have demonstrated the utility of T-RFLP, RT-PCR, and qPCR and the potential utility of Prokaryotic cDNA Subtraction to improve our ability to monitor and understand perchlorate reduction in a biological treatment system.

We examined the microbial community present in a pilot-scale FXB reactor for perchlorate reduction (operated by Carollo Engineers in Rialto, California as part of ESTCP project ER-0544: Direct Fixed-Bed Biological Perchlorate Destruction Demonstration) by culture-based and culture-independent methods. While culturing showed *Dechlorospirillum* as the only PRB in the reactor, T-RFLP showed a more diverse community of PRB including *Dechloromonas* and other members of the *Rhodocyclaceae* family, as well as *Ideonella dechloratans* (a chlorate-reducing bacterium). It is also possible that other populations observed in the T-RFLP profiles are PRB that are not currently recognized as such. The purpose of examining the microbial community in the pilot-scale reactor was to determine if the known PRB found in that reactor were those for which functional gene sequences related to perchlorate reduction (e.g., *pcrA* and *cld*) were already available. *Dechloromonas* and *Dechlorospirillum*, both found in the reactor, have complete or partial sequences published for *pcrA* or *cld*. However, the reactor might contain other populations (e.g., other members of the *Rhodocyclaceae* family) with unsequenced genes related to perchlorate reduction. Thus, the next step of the project addressed the need to expand the limited database of sequences related to perchlorate reduction.

Our group has recently published the first application of Prokaryotic cDNA Subtraction (De Long et al., 2008), in which this functional gene discovery technique was used to interrogate an organism with a fully sequenced genome (*Pseudomonas putida* mt-2). In our current project, we applied Prokaryotic cDNA Subtraction to PRB strain JDS4, which was the first time Prokaryotic cDNA Subtraction had been applied to a microorganism with an unsequenced genome. Our goal was to isolate and sequence the genes related to perchlorate reduction for this strain and compare them with the other (few) published sequences related to perchlorate reduction. None of the genes identified were related to perchlorate reduction, and ninety-seven percent of the genes identified were rRNA genes. While the current protocol for Prokaryotic cDNA Subtraction is not sufficient to isolate genes related to perchlorate reduction in environmental PRB JDS4 (likely due to the presence of too much residual rRNA in the purified mRNA), we are pursuing several ways to rectify this situation. We are in discussions with Ambion, Inc. and Clontech Laboratories, as they have both expressed interest in contributing to our on-going improvement of Prokaryotic cDNA Subtraction.

Since no new sequences related to perchlorate reduction were identified by Prokaryotic cDNA Subtraction, we used primer pairs targeting *pcrA* and *cld* that already exist in the literature (Nozawa-Inoue et al., 2008; Bender et al., 2004) for the rest of the project.

While the presence of genes related to perchlorate reduction has been measured by several research groups, to our knowledge, we are the first to have measured transcripts related to

perchlorate reduction. Our data show that active biological perchlorate reduction correlates to the expression of *cld*. While *cld* was strongly expressed under perchlorate-reducing conditions, it was not expressed under aerobic conditions; furthermore, *cld* expression was quickly curtailed under anoxic conditions. Thus, transcriptional data will be useful to provide an additional line of evidence for biological perchlorate reduction and to gauge the activity of PRB in a process. Given the utility of this type of data, we are extending the *pcrA* qPCR assay of Nozawa-Inoue et al. (2008) to develop an RT-qPCR assay; we have also developed a qPCR assay for *cld* and will extend that to an RT-qPCR assay. We will apply these quantitative assays to our bench-scale FXB reactors for perchlorate reduction to demonstrate the correlation between perchlorate reduction and transcription of genes related to perchlorate reduction under different water quality conditions.

The combination of MBT used in this project allowed us to assess the composition of the microbial community during active perchlorate reduction as well as to detect the presence and expression of genes associated with perchlorate reduction. Therefore, this combination of MBT can be used to provide an additional line of evidence for biological perchlorate reduction, to gauge the activity of PRB in a process, and perhaps act as an early warning system for upsets to the PRB community.

Our transition plan is to build upon the results from this SEED project by demonstrating the utility of RT-qPCR in our bench-scale FXB reactors and at a field site. In addition, we strongly believe in expanding the limited database of gene sequences related to perchlorate reduction, and we plan on modifying our Prokaryotic cDNA Subtraction protocol to render it successful for PRB; this will be done with partial assistance from Ambion, Inc. and Clontech Laboratories.

Appendices

List of Technical Publications

Conference Papers

Kirisits, M. J., S. K. De Long, K. A. Kinney, S. Desai, and J. C. Brown. 2008. Characterizing the Microbial Community for a Bioreactor Treating Perchlorate-Contaminated Groundwater. *Proceedings of the 2008 Inorganic Contaminants Workshop*. Albuquerque, New Mexico.

De Long, S. K., M. J. Kirisits, and K. A. Kinney. 2008. Perchlorate-Reducing Gene Targets for Bioremediation Applications. *Proceedings of the Sixth International Conference on Remediation of Chlorinated and Recalcitrant Compounds*. Monterey, California.

Published Technical Abstracts

Kirisits, M. J., K. A. Kinney, and S. K. De Long. 2007. Characterizing the Microbial Community in a Bioreactor Treating Perchlorate-Contaminated Groundwater. *Abstracts of the Partners in Environmental Technology Technical Symposium and Workshop.* Washington, D.C.

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